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TITLE: Homocysteine Is an Oncometabolite in Breast Cancer, Which Promotes Tumor Progression and Metastasis

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14. ABSTRACT The goal of this project is to interrogate the role of the amino acid homocysteine in breast cancer progression and metastasis and to test the hypothesis that this amino acid is actually an oncometabolite. According to this hypothesis, homocysteine levels increase in breast cancer, which results in changes in gene expression in tumor cells helping the tumors to grow and metastasize. The molecular basis for the increase in the levels of this amino acid in breast cancer is the downregulation of the enzyme methylene tetrahydrofolate reductase (MTHFR) via DNA methylation. This enzyme is responsible for the conversion of methylene tetrahydrofolate to methyl tetrahydrofolate, which is necessary to convert homocysteine into methionine by serving as a cofactor for methionine synthase. As such, the downregulation of MTHFR in breast cancer reduces the activity of methionine synthase and thus interferes with the conversion of homocysteine into methionine, thus increasing the levels of homocysteine. We hypothesize that homocysteine promotes Wnt/beta-catenin signaling, increases IL-6, TGF-beta, ANGPTL4, and MMP9 expression, thus driving tumor progression at the primary site and also promoting metastasis of the cancer to the lungs.					
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1. Introduction

The goal of this project is to interrogate the role of the amino acid homocysteine in breast cancer progression and metastasis and to test the hypothesis that this amino acid is actually an oncometabolite. According to this hypothesis, homocysteine levels increase in breast cancer, which results in changes in gene expression in tumor cells helping the tumors to grow and metastasize. The molecular basis for the increase in the levels of this amino acid in breast cancer is the downregulation of the enzyme methylene tetrahydrofolate reductase (MTHFR) via DNA methylation. This enzyme is responsible for the conversion of methylene tetrahydrofolate to methyl tetrahydrofolate, which is necessary to convert homocysteine into methionine by serving as a cofactor for methionine synthase. As such, the downregulation of MTHFR in breast cancer reduces the activity of methionine synthase and thus interferes with the conversion of homocysteine into methionine, thus increasing the levels of homocysteine. We hypothesize that homocysteine promotes Wnt/beta-catenin signaling, increases IL-6, TGF-beta, ANGPTL4, and MMP9 expression, thus driving tumor progression at the primary site and also promoting metastasis of the cancer to the lungs.

2. Keywords

MTHFR - methylene tetrahydrofolate reductase

IL-6 – interleukin-6

TGF-beta – Transforming growth factor beta

ANGPTL4 – Angiopoietin-like 4

DNMT – DNA methyl transferase

3. Accomplishments

Goals of the project

1. Investigate using two different mouse models of spontaneous breast cancer (MMTV-HRAS mouse and MMTV-PyMT mouse) whether *Mthfr* is silenced through DNA methylation and as a result the levels of the oncometabolite homocysteine are elevated in tumors.
2. Investigate whether homocysteine promotes breast cancer progression and lung metastasis by comparing the disease process in MMTV-HRAS and MMTV-PyMT mice on two different genetic backgrounds: *Mthfr*^{+/+} and *Mthfr*^{-/-}. Investigate the ability of homocysteine to induce TGF-β, ANGPTL4, and MMP-9 in breast cancer cell lines and to disrupt the barrier function of lung microvascular endothelial cells.
3. Investigate using breast cancer cell lines whether over expression of MTHFR or exposure to *N*₅-methyltetrahydrofolate decreases cell proliferation in vitro and suppresses tumor growth in xenografts in vivo.

Goals accomplished

There were two tasks scheduled to be completed during the first 12 months of the funding period. First was to determine the circulating and tissue levels of homocysteine in wild type mice and in two different mouse models of spontaneous breast cancer. We have completed this study. We found the levels of homocysteine to be increased 4.5-fold in MMTV-HRAS mouse breast tumor tissues compared to age-matched wild type mouse mammary tissues. Similarly, the levels of homocysteine went up 7.3-fold in MMTV-PyMT mouse breast cancer tissues

compared to age-matched wild type mouse mammary tissues. Recently, we have established two additional mouse models of spontaneous breast cancer: MMTV-Neu transgenic mouse and C3(1)-SV40 transgenic mouse. We will measure homocysteine levels in tumor tissues from these mice to determine if the observations made in the other two mouse models hold true for these two new mouse models. The plasma samples have been collected from all of these mice. We are now running the HPLC to determine the circulating levels of homocysteine in these mice. This task also included determination of expression of Mthfr and Dnmts (Dnmt1, Dnmt3a and Dnmt3b) in breast tumor tissues and compare the expression levels in normal mouse mammary gland. For this, we used biological triplicates by preparing RNA from tumor tissues from three different mice for each genotype. We have analyzed the expression levels of these genes by RT-PCR. We found that the expression of Mthfr was almost completely abolished in breast cancer tissues in all four models (MMTV-Hras-Tg, MMTV-Neu-Tg, MMTV-PyMT-Tg, and C3(1)-SV40-Tg) (Fig. 1). This downregulation of Mthfr expression was correlated with upregulation of the DNA methyltransferases Dnmt1 and Dnmt3b. These data provide strong evidence for our original hypothesis that MTHFR is silenced in breast cancer due to increased DNA methylation and that the downregulation of MTHFR increases the tissue levels of homocysteine. This finding is central to the core hypothesis of the funded project implicating homocysteine as an oncometabolite in breast cancer. Western blots are being planned with tissue samples from these mice to confirm that observed increase in mRNA for Dnmt1 and Dnmt3b and the decreased mRNA for Mthfr translate into corresponding changes in protein levels.

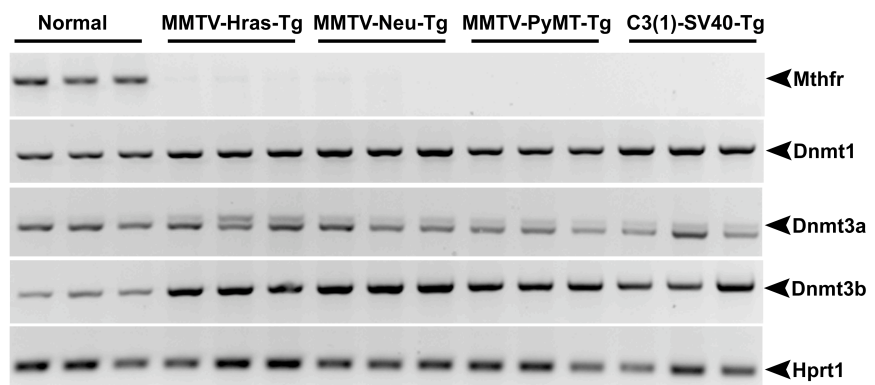


Fig. 1. Expression analysis of Mthfr, Dnmt1, Dnmt3a and Dnmt3b in normal mammary gland and in breast tumor tissues collected from four different mouse models of spontaneous mammary tumors.

The second task was to generate MMTV-Hras/Mthfr^{-/-} and MMTV-PyMT/Mthfr^{-/-} mouse lines to determine the consequences of Mthfr deletion on breast cancer progression. We have accomplished this goal. We now have these mouse lines and confirmed their genotype using tail genomic DNA.

Plans for the next reporting period

1. We will complete the measurement of plasma levels of homocysteine using the samples that have been collected and stored from control mice and transgenic mice with breast tumors. We will also complete the western blots for the assessment of protein levels for Mthfr and the different isoforms of Dnmts.
2. We will monitor incidence, metastasis, and survival of MMTV-Hras/Mthfr^{+/+} mice, MMTV-Hras/Mthfr^{-/-} mice, MMTV-PyMT/Mthfr^{+/+} mice, and MMTV-PyMT/Mthfr^{-/-} mice.
3. We will collect mammary tumor tissues from the mice of all these four genotypes and measure homocysteine levels. We will also collect blood samples for measurement of homocysteine in plasma.

3. Impact

Confirmation that Mthfr is indeed silenced in four different mouse models of spontaneous breast cancer has profound impact on the biology of breast cancer. There is little literature evidence implicating homocysteine as a driver of cancer progression. But there has been lots of public attention to the potential contribution of this amino acid to cardiovascular diseases and also a pregnancy disorder known as preeclampsia. Polymorphisms in MTHFR that decrease the catalytic activity of the enzyme are common in the general population and these polymorphisms result in significant increase in plasma levels of homocysteine. Our findings show that the expression of the enzyme is almost completely suppressed in breast cancer tissues in mice and as a consequence homocysteine levels increase in tumor tissues. If the planned studies in the project demonstrate as hypothesized that homocysteine enhances breast cancer progression and metastasis, it will implicate polymorphisms in MTHFR in potentiating breast cancer growth, progression, and metastasis. These findings also have another important aspect related to public health. Plasma homocysteine levels are also increased when dietary intake of three vitamins, folic acid, vitamin B12, and pyridoxine, is low. Therefore, the nutritional status of individuals in terms of these three vitamins will also have an impact on the growth, progression, and metastasis of breast cancer.

Impact on other disciplines: None

Impact on technology transfer: None

Impact on society beyond science and technology: As indicated above, screening of blood for homocysteine levels might be useful in predicting the aggressiveness of the breast cancer. If certain breast cancer patients have elevated levels of homocysteine indicating a more aggressive growth of the tumor, therapeutic steps can be taken by the patients to take vitamin supplements to improve their nutritional status of folic acid, vitamin B12, and pyridoxine so as to reduce the homocysteine levels and hence suppress breast cancer growth, progression and metastasis.

5. Changes/Problems

There are no changes or problems in the execution of the planned experiments. The only issue is that the grant is going to be transferred to another institution (from Georgia Regents University to Texas Tech University Health Science Center) because of the relocation of the Principal Investigator's laboratory. This might delay progress in the project a little bit because of the practical aspects associated with the shifting of the laboratory, mice, and personnel, but will not interfere with the progress in the project in the long run. We still believe that all of the experiments proposed in the funded project will be completed on time within the proposed project period.

6. Products

None

7. Participants & other collaborating organizations

Vadivel Ganapathy	Principal Investigator
Effort	1.2 months
Contribution	Overall control of the project and design of the experiments

Sylvia B. Smith	Co-Investigator
Effort	1 month
Contribution	Maintenance of Mthfr-null mice, tissue and blood collection
Muthusamy Thangaraju	Co-Investigator
Effort	1 month
Contribution	Mouse genotyping, generation of transgenic mice on Mthfr-null background
Puttur D. Prasad	Co-Investigator
Effort	1 month
Contribution	Homocysteine measurements
Yangzom D. Bhutia	Research Associate
Effort	5 months
Contribution	RT-PCR, q PCR, and western blots for Mthfr and Dnmts

Other support

There have been no changes in “other support” for any of the key personnel in the project.

8. Special reporting requirements

None

9. Appendices

None